

Attorney Docket No.: RU-0064
Inventors: Lazarus et al.
Serial No.: 09/332,886
Filing Date: June 15, 1999
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Amendments to the Specification:

Please replace the paragraph beginning at line 9 of page 9 with the following:

FIG. 3 shows schematically a proposed model for 2,5-DKG reductase A (SEQ ID NO:1).

Please replace the paragraph beginning at line 15 of page 26 with the following:

An aliquot of plasmid ptrpl-35 was digested with *EcoRI* and *HindIII* restriction enzymes and the resulting 1690 base pair fragment purified by agarose gel electrophoresis. This fragment was then ligated into *EcoRI* and *HindIII* digested vector M13 mp19. The resulting recombinant phage (called M13 mp19.DKGRA) was used to isolate a single stranded template form of the phage for subsequent mutagenesis. The template was mutagenized with three oligonucleotides to introduce three new restriction enzyme cleavage sites to the 2,5-DKG reductase A gene. These sites were all 'silent' in that although they introduce a new restriction cleavage site to the DNA sequence, the amino acid sequence of the protein coded for remains unchanged, due to degeneracy in the genetic code. The three mutagenic oligonucleotides and the changes introduced are as follows: 1) oligonucleotide *XbaA* has

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sequence 5' CGCGAAGCTGGCTCTAGATCAGGTCGAC 3' (SEQ ID NO:2) and introduces a new *Xba*I site at amino acid position 98; 2) oligonucleotide *Apa*A has sequence 5' ATCGTGGGGGCCCCTCGGTCAGGGC 3' (SEQ ID NO:3) and introduces a new *Apa*I site at amino acid position 188; and 3) oligonucleotide *Kpn*A has sequence 5' GAGGTCGACTGAGGTACCCGAACACCCG 3' (SEQ ID NO:4) and introduces a new *Kpn*I site immediately following the stop codon (TGA) after the final amino acid. The mutagenesis reaction and conditions were essentially the same as described in Example 2 for the construction of mutant Q192R. After the mutagenesis reaction, ~~positives~~ positive plaques were identified by hybridization to the mutagenic oligonucleotide under stringent conditions, and the entire coding region of the 2,5-DKG reductase A fragment was sequenced to confirm the mutations.

Please replace the paragraph beginning at line 8 of page 29 with the following:

A synthetic oligonucleotide with the sequence 5' GCCCCTCGGTCGCGCAAGTACG 3' (SEQ ID NO:5) was synthesized and phosphorylated as follows: the oligonucleotide was diluted to a concentration of 5.0 OD₂₆₀ units per ml. Then 2.5 µl of oligonucleotide was combined with 3 µl 10x kinase buffer (1 M

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tris pH 8.0, 100 mM MgCl₂, 70 mM dithiothreitol, 10 mM ATP), 25 µl water, and 2 units of T4 polynucleotide kinase (New England Biolabs). The mixture was incubated at 37°C for 15 minutes, then the kinase enzyme was inactivated by heating to 70°C for 10 minutes.

Please replace the paragraph beginning at line 16 of page 33 with the following:

A mutant form of 2,5-DKG reductase A was discovered which, although having activity equivalent to the wild-type enzyme, had increased amounts of the protein accumulating in the *Acetobacter* expression host. This mutant, named "HS1", contains three amino acid changes: asparagine replaces threonine at position two, threonine replaces serine at position five, and serine replaces valine at position seven. The synthesis of this mutant was directed by a 37 base oligonucleotide with the sequence 5' AATTCTATGAACGTTCCCACCATCAGCCTCAACGAC 3' (SEQ ID NO:6). The steps in the mutagenesis reaction were essentially the same outlined for construction of the Q192R mutant.

Please replace the paragraph beginning at line 9 of page 35 with the following:

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A mutant form of 2,5-DKG reductase A is discovered which has increased temperature stability in the *Acetobacter* expression host. This mutant contains two amino acid changes: alanine replaces glycine at position 55, and alanine replaces glycine as position 57. The synthesis of this mutant is directed by a base oligonucleotide with the sequence 5' GAAACGAAGAAGCGGTCGCGGCCGCGATCGCG 3' (SEQ ID NO:7). The steps in the mutagenesis reaction are essentially the same as outlined for the construction of the Q192R mutant.

Please replace the paragraph beginning at line 23 of page 35 with the following:

Mutant forms of the 2,5-DKG reductase A were discovered which showed major reductions in activity for converting 2,5-DKG to 2-KLG. The steps in the mutagenesis reactions were essentially the same as outlined for construction of the Q192R mutant. The following base oligonucleotides directed the synthesis of such mutants showing reduced activity in the *Acetobacter* expression host: with a substitution of alanine for glycine at position 191 to construct the G191A mutant, 5' GGGGCCGCTCGCCCAGGGCAAGT 3' (SEQ ID NO:8); with a deletion of glycine at position 193 to construct the G193 deleted mutant, 5'

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CCGCTCGGTCAGAAGTACGACCT 3' (SEQ ID NO:9); with a substitution of arginine for lysine at position 194 to construct the K194R mutant, 5' CGGTCAGGGCCGCTACGACCTCT 3' (SEQ ID NO:10); with a substitution of serine for tyrosine at position 195 to construct the Y195S mutant, 5' TCAGGGCAAGTCGGACCTCTTCG 3' (SEQ ID NO:11); with a substitution of tyrosine for alanine at position 167 to construct the A167Y mutant, 5' GCTGCACCCCTACTACCAGCAGC 3' (SEQ ID NO:12); with a substitution of phenylalanine for tyrosine at position 168 to construct the Y168F mutant, 5' GCACCCCGCCTTCCAGCAGCGCG 3' (SEQ ID NO:13); with a substitution of proline for glutamine at position 169 to construct the Q169P mutant, 5' CCCC GCCTACCCGCAGCGCGAGA 3' (SEQ ID NO:14); with a substitution of leucine for lysine at position 225 to construct the K225L mutant, 5' GCACCTGCAGCTCGGTTTCGTGG 3' (SEQ ID NO:15); with a substitution of serine for phenylalanine at position 227 to construct the F227S mutant, 5' GCAGAAGGGTTCGGTGGTCTTCC 3' (SEQ ID NO:16); with a substitution of threonine for valine at position 228 to construct the V228T mutant, 5' GAAGGGTTTCACCGTCTTCCCGA 3' (SEQ ID NO:17); with a substitution of proline for valine at position 229 to construct the V229P mutant, 5' GGGTTTCGTGCCCTTCCCGAAGT 3' (SEQ ID NO:18); and with a stop

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codon at position 271 to construct the truncation mutant, 5'

GGGTCGCGTGTGAGCACACCCCG 3' (SEQ ID NO:19).